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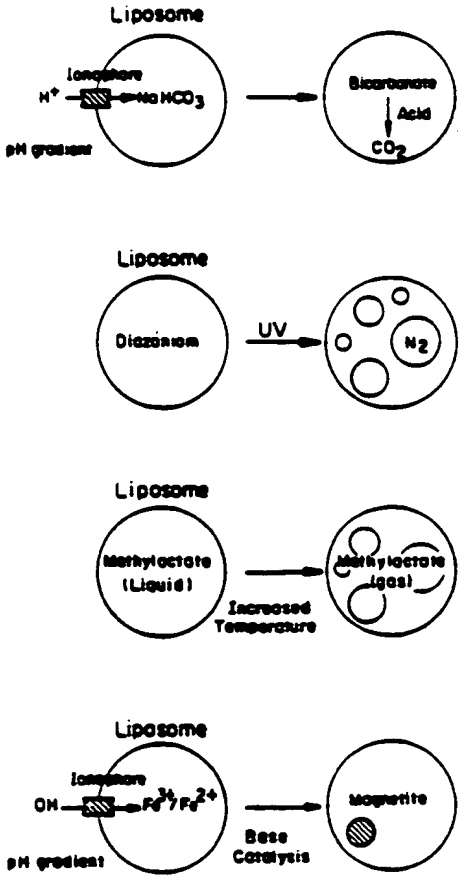
(54) Title: LIPOSOMES AS CONTRAST AGENTS FOR ULTRASONIC IMAGING

(57) Abstract

Liposomes suitable as ultrasound contrast agents which contain media of various types including gases, gaseous precursors activated by pH, temperature or pressure, as well as other solid or liquid contrast enhancing agents, are described. Methods of using the same as ultrasound contrast agents are also disclosed. The present invention also comprises novel methods for synthesizing liposomes having encapsulated therein gases.



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"Liposomes as contrast agents for ultrasonic imaging."

RELATED APPLICATION

This application is a continuation-in-part of copending application U.S. Serial No. 455,707, filed December 22, 1989.

BACKGROUND OF THE INVENTION

Field Of The Invention

This invention relates to the field of ultrasonic imaging, and, more specifically, to the use of liposomes in ultrasonic imaging procedures.

Background Of The Invention

There are a variety of imaging techniques which have been used to detect and diagnose disease in animals and humans. One of th first techniques us d f r diagnostic imaging was X-rays. The images btained thr ugh this technique r flect the lectr n d nsity of the bject being imaged. Contrast agents such as barium or iodine are used to attenuate r block X-rays such that the c ntrast between

various structures is increased. For example, barium is used for gastrointestinal studies to define the bowel lumen and visualize the mucosal surfaces f the bowel. Iodinated contrast media is used intravascularly to visualize the arteries and this is called angiography. X-rays, however, are known to be dangerous. The radiation employed in X-rays is ionizing and the deleterious effects of the ionizing radiation are cumulative.

Magnetic resonance imaging (MRI) is another important imaging technique, however it has the drawbacks of expense and the fact that it cannot be conducted as a portable examination. In addition, MRI is not available at many medical centers.

Radionuclides, employed in nuclear medicine, provide another imaging technique. In employing this technique, radionuclides such as technetium labelled compounds are injected into the patient, and images are obtained from gamma cameras. Nuclear medicine techniques, however, suffer from poor spatial resolution and expose the animal or patient to the deleterious effects of radiation. Furthermore, there is a problem with the handling and disposal of radionuclides.

Ultrasound, another diagnostic imaging technique, is unlike nuclear medicine and X-rays in that it does not expose the patient to the harmful effects of ionizing radiation. Moreover, unlike magnetic resonance imaging, ultrasound is relatively inexpensive and can be conducted as a portable examination. In using the ultrasound technique, sound is transmitted into a patient or animal via a transducer. When the sound waves propagate through the body, they encounter interfaces from tissues and fluids. Depending on the reflectivity and acoustic properties of the tissues and fluids in the body, the ultrasound sound waves are either reflected or absorbed. When sound waves are reflected by an interfac they are d tect d by th receiver in the transducer and pr cessed t form an image. The ac ustic pr p rties of the tissues and fluids within the body det rmin the contrast which app ars in th r sultant imag .

Advances have been made in recent years in ultrasound technology. However, despite these various techn logical improvements, ultrasound is still an imperfect tool in a number of respects, particularly with respect to the detection of disease in the liver and spleen, kidneys and vasculature and in measuring blood flow. The ability to detect and measure these things depends on the difference in acoustic properties between blood or other tissues and the surrounding tissues. As a result, contrast agents have been sought which will increase the acoustic difference betw n blood and surrounding tissues in order to improve the measurement of blood flow, or between one tissue and another such as between the liver and a tumor in order to improve disease detection.

The principles underlying image formation in ultrasound have directed researchers to this pursuit of contrast agents. When sound waves from ultrasound pass through a substance, the acoustic properties of that substance will depend upon the velocity of the sound and the density of that substance. Changes in the acoustic properties or acoustic impedance of the substance are most pronounced at interfaces of different substances with greatly different density or acoustic impedance, particularly at the interface between solids, liquids and gases. When the ultrasound sound waves encounter such interfaces, the changes in acoustic impedance result in a more intense reflection of sound waves and a more intense signal in the ultrasound image.

Many of the prior art contrast agents developed to date for ultrasound have comprised liquids containing microbubbles of gas where the microbubbles have b en encapsulated with gelatin or saccharine. Those microbubble and gelatin/saccharine constructs have most often been prepared using agitation techniques. Other prior art is directed t attempts with prot in-associated air bubbles or air bubbl s inc rp rated in microspheres composed of either albumin or c llagen. Furthermore, h avy m tal particulates have b n evaluated as ultrasound contrast agents. There

have als been s me reports f liposomes described as useful in ultrasonic applications having gas or gaseous precursors encapsulated therein.

While the prior art has produced some ultrasound contrast agents which are echogenic on ultrasound, that is, provide a contrast enhancement signal, the contrast agents developed thus far have various problems. The protein based air bubble systems have the drawback that a foreign protein which may be antigenic and potentially toxic is being employed. The liposomal contrast agents have had problems with uneven size distribution and poor stability. The gaseous precursor containing liposomes have also been inefficient in their ability to form contrast enhancing gas in vivo. Moreover, while some of the prototype prior art contrast agents have demonstrated echogenic effects as transpulmonary vascular contrast agents, many of these agents have failed to demonstrate a convincing effect on improving tumor imaging in, for example, the liver or spleen. Furthermore, many of th methods for preparing these ultrasound contrast agents, particularly the gas encapsulated liposomes, are inefficient, expensive, and otherwise unsatisfactory.

The present invention is directed to answering these and other important needs.

SUMMARY OF THE INVENTION

In one embodiment, the present invention is directed to a contrast agent for ultrasonic imaging comprising an ionophore-containing liposome having encapsulated therein a pH-activated gaseous precursor.

In another embodiment, the present invention is directed to a contrast agent for ultrasonic imaging which comprises a liposome having encapsulated therein a photo-activated gaseous precursor.

In a third mb diment, the present invention is directed to a c ntrast agent f r ultrasonic imaging which comprises a liposome having ncapsulated th rein a t mperatur -activated gaseous pr cursor.

In a further embodiment, the present invention is directed to a contrast agent for ultrasonic imaging which comprises a liposome having encapsulated therein a solid or liquid contrast enhancing agent.

An even further embodiment of the invention is directed to a method for imaging a patient using ultrasound comprising administering to the patient a liposome of the invention and scanning the patient using ultrasound.

In a still further embodiment, the present invention comprises novel methods for encapsulating a gas within the internal space of a liposome to produce contrast agents for ultrasonic imaging.

The contrast agents embodied within the present invention are echogenic, that is, capable of reflecting ultrasound waves to enhance signal intensity on an ultrasound image. In certain preparations particularly designed as intravascular contrast agents, the present contrast agents are small enough to pass through the capillaries of pulmonary circulation and are effective in providing good contrast enhancement of the heart, arterial system and venous system. In other preparations designed for injecting into other structures or cavities, the vesicles are larger to maximize echogenicity and provide highly effective contrast enhancement. In accordance with the present invention, the liposomes with the gas, gaseous precursors and/or solid or liquid contrast enhancing agents encapsulated therein can be produced in defined and reproducible sizes. The present invention also allows targeting and delivery of the contrast agent to specific sites such as the vasculature, liver, spleen and kidney. The present invention is free from the toxicity associated with the use of foreign proteins to encapsulate air bubbles and also minimizes the likelihood of embolisms occurring. In addition, the liposomes of the present invention are capable of long term storage. Moreover, the novel methods of the invention for encapsulating gas within the internal space of liposome are highly efficient and inexpensive to carry out.

The liposomal ultrasound contrast agents of the invention permit advanced imaging f rgans and tissues in a way not pr viously contemplated. Because lip s me membranes can be optimized for blood pool or circulation half-life, effective perfusion and blood pool contrast agents will be available. This will be useful in the heart, for example, for diagnosing ischemia and in other organs for diagnosing decreased blood flow or shunts. In blood pool lesions such as cavernous hemangioma, these agents will be useful for making accurate diagnoses. Because these agents can be optimized for uptake by healthy cells in organs such as th liver and spleen, the contrast agent facilitates the ultrasonic detection and characterization of tumors in these organs.

These and other features of the invention and the advantages thereof will be further described in the drawings and description below.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A. In this figure, the general method in which a gaseous precursor reacts to form a gas in response to a change in pH within a vesicle is described. In the example illustrated, bicarbonate salts are entrapped within the interior aqueous space of the vesicle and an ionophore such as, for example, p-trifluoromethoxy- carbonylcyanide phenylhydrazone is present within the liposome membrane matrix to promote hydrogen ion flux across the liposomal membrane. The pH change within the vesicle interior facilitated by th ionophore results in the formation of a highly echogenic carbon dioxide gas.

Figure 1B. In this figure, the general method in which a gaseous precursors reacts to form gases upon exposure to UV light is described. In the specific example illustrated, diazonium c mpounds trapped insid the lipid vesicles form a highly echogenic nitrogen gas as a result of UV exposure.

Figure 1C. In this figur , th general m thod in

which a gaseous precursor forms a gas in response to an increase in temperature, is illustrated. Once inject d into a patient, methylactate, for example, is transformed from a liquid to a highly echogenic gas as a result of the increase in temperature from room temperature to physiological temperature.

Figure 1D. In this figure, one method of entrapping a particulate solid contrast enhancing agent such as magnetite, within a liposome is described. In the illustrated method, a mixture of ferrous and ferric salts is entrapp d within the aqueous core of the liposome. An ionophore such as valinomycin is incorporated within the matrix of the liposome in order to increase the rate of proton flux across the membrane. Prior to or during use, the pH on the exterior of the vesicle is then increased by the addition of the appropriate alkali resulting in an increase in the pH in the interior of the liposome. The increase in pH in turn promotes base catalysis which results in the in situ formation of highly echogenic magnetite within the liposome. It is equally possible to entrap preformed solid contrast enhancing agents such as preformed magnetite in the liposomes.

Figure 2. In this figure is illustrated one method of modifying the surface of a liposome with polymer such as polyethylene glycol (PEG), so as to be able to modulate the clearance and biodistribution kinetics of the liposomes with entrapped gas, gaseous precursors and/or solid or liquid contrast enhancing agents.

Figure 3. This figure illustrates the basic pressurization and depressurization phenomenons behind some of the devices and methods for preparing the gas-containing liposomes of the invention. First, liposomes are added t a vessel, and the vessel is then pressurized with gas. Under pressure, the gas goes into solution and passes across the liposom membranes. When th pr ssure is released, gas bubbl s f rm within the lipos mes.

Figure 4. This figure illustrat s on apparatus of th inv ntion for synth sizing liposom s having

encapsulated therein a gas. Th apparatus is utilized by placing a liquid media which contains liposomes into the v ss l. A cap is then threaded ont the vessel pening providing a pressure tight seal. The vessel is pressurized by fitting a cartridge containing a gas, such as carbon dioxide, into an inlet port. The cartridge discharges its contents into the upper end of a tube fitted into the vessel. The gas flows through the tube and exits at the lower end of the tube into the bottom of the vessel. After the gas has been introduced into the vessel, the vessel can then be depressurized by ejecting the liquid from the vessel.

Figure 5. This figure illustrates another apparatus of the invention for synthesizing liposomes having encapsulated therein a gas. A syringe in which liposomes have been placed is connected via one or more filters of various pore sizes to an inlet/outlet port and valve of the pressur vessel. The syringe is then emptied through the filters and the inlet/outlet port and valve into the bottom of the vessel. Alternatively, the vessel may be directly loaded with the liposomes without using the syringe and/or filters, and/or inlet/outlet port and valve. The vessel is then pressurized with a gas, resulting in a gas-containing lipos me composition. The gas-containing liposome contents of the vessel may then be discharged through the inlet/outlet port and valve and the filter assembly, and emptied into the syringe. Alternatively, the liposome may be removed directly without passing through the filter and/or inlet/outlet port and valve and/or emptying into the syringe.

Figure 6. This figure illustrates a further apparatus of the invention for synthesizing liposomes having encapsulated therein a gas. The gas enters a vessel in which a liquid media containing liposomes has been placed through an inlet port, flows through a tube and discharges into the b ttom of the vess l. From th b tt m f th v ssel, th gas bubbles upward thr ugh the liquid. Depressurization is acc mplish d by pening a valve n a s parat utlet port, thereby j cting the liquid from the bott m of the v ssel

through the tube.

Figure 7. This figure illustrates another apparatus for synthesizing liposomes having encapsulated therein a gas. As this figure illustrates, the apparatus required to practice the method of the invention need only be a simple vessel with a port for introducing and discharging the pressurized gas and liposomes.

Figure 8. This figure illustrates another apparatus for synthesizing liposomes having encapsulated therein a gas. This apparatus is utilized by placing a liquid media which contains liposomes in a vessel jacketed by a chamber through which a coolant circulates. A high frequency sound wave generator is attached to the vessel. In use, the vessel is pressurized using a gas introduced through a gas port. The sound wave generator transforms electrical energy into mechanical oscillations and transmits the oscillations into the liquid through a horn which extends into the vessel. As the liposomes break up and reform as a result of the mechanical forces, they encapsulate the dissolved gas within their internal aqueous core. Following sonication, the vessel is depressurized and the encapsulated gas forms bubbles, thereby transforming the liposomes into gas-containing liposomes.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the present invention provides a contrast agent for ultrasound imaging which comprises an ionophore-containing liposome having encapsulated therein a pH-activated gaseous precursor.

As used herein, the phrase "ionophore-containing liposome" denotes a liposome having incorporated in the membrane thereof an ionophore. The term "ionophore", as used herein, denotes compounds which are capable of facilitating the transport of hydrogen ions or hydroxide ions across the liposome membrane to effect a change in pH inside the liposome membrane, and includ compounds commonly ref rr d to as proton carriers and channel formers. Suitable ionophores include

proton carriers such as nitro-, halo- and oxygenated phenols and carbonylcyanide phenylhydrazones. Preferred of such proton carriers are carbonylcyanide, p-trifluoromethoxyphenylhydrazone (FCCP), carbonylcyanide M-chlorophenylhydrazone (CCCP), carbonylcyanide phenylhydrazine (CCP), tetrachloro-2-trifluoromethyl benzimidazole (TTFB), 5,6-dichloro-2-trifluoromethyl benzimidazole (DTFB), and Uncoupler 1799 Suitable channel formers include gramicidin, alamethicin, filipin, etruscomycin, nystatin, pimaricin, and amphotericin. Other suitable proton carriers include the following compounds which preferably exhibit selectivity for cations, but will also transport protons and/or hydroxide ions: valinomycin, enniatin (type A, B or C), beauvericin, monomycin, nonactin, monactin, dinactin, trinactin, tetranactin, antamanide, nigericin, monensin, salinomycin, narisin, mutalomycin, carriomycin, dianemycin, septamycin, A-204 A, X-206, X-537 A (lasalocid), A-23187 and dicyclohexyl-18-crown-6. Such ionophores are well known in the art and are described, for example in Jain et al., Introduction to Biological Membranes, (J. Wiley and Sons,. N.Y. 1980), especially pp. 192-231, and Methyl Ions In Biological Systems, ed. H. Sygel, Vol. 19, "Antibiotics And Their Complexes" (Dekker, N.Y. 1985), disclosures of each of which are incorporated herein by reference in their entirety. The ionophores may be used alone or in combination with one another.

It has been found that although liposomes are not impermeable to protons or hydroxide ions, the permeability coefficient of liposomes is generally so very low that it often takes weeks or months to dissipate a pH gradient. Providing a more rapid transport of hydrogen ions or hydroxide ions across a liposome membrane in order to activate pH-modulated gaseous precursors is necessary. The incorporation of ionophores in the liposome membrane, in accordance with the present invention, provides the necessary means of transporting the activating ions. By increasing the rate of hydrogen or hydroxide ion flux across the liposome membrane,

such ionophores will increase the rat within the liposome of gas formation from the pH-activated gaseous precursor. This phenomenon is diagrammatically r present d in Figur 1A.

The phrase "pH-activated gaseous precursor", as used herein, denotes a compound in solid or liquid form which, when exposed to a drop in pH, will form a gas. As noted above, this concept is illustrated in Figure 1A. Such compounds include, but are not limited to, metal carbonate and bicarbonate salts, such as the alkali metal carbonates and bicarbonates, and the alkaline earth carbonates and bicarbonates, and mixtures thereof. Exemplary of such compounds are lithium carbonate, sodium carbonate, potassium carbonate, lithium bicarbonate, sodium bicarbonate, potassium bicarbonate, magnesium carbonate, calcium carbonate, magnesium bicarbonate, and the like. Also useful gas generating compounds are ammonium carbonate, ammonium bicarbonate, ammonium sesquecarbonate, sodium sesquecarbonate, and the like. These compounds, when dissolved in water, show a pH of greater than about 7, usually between about 8 and about 12. Other pH-activated gaseous precursors include aminomalonate, which, when dissolved in water, generally shows a pH of about 5 to 6.

The pka1 of aminomalonate is 3.32 and the pka2 is 9.83. Aminomalonate is well known in the art, and its preparation is described, for example, in Thanassi, Biochemistry, Vol. 9, no. 3, pp. 525-532 (1970), Fitzpatrick et al., Inorganic Chemistry, Vol. 13, no. 3, pp. 568-574 (1974), Stelmashok et al., Koordinatsionnaya Khimiya, Vol. 3, no. 4, pp. 524-527 (1977). Other suitable pH-activated gaseous precursors will be apparent to those skilled in the art.

As those skilled in the art would recognize, such compounds can be activated prior to administration, if desired. Of course, by choosing a gaseous precursor with the appr priate pKa, on skilled in th art can prepare a liposome wher by gas will form in th lipos me after intrav nous inj ction r injecti n into a body cavity. Ev n when exp sure to the appropriat pH occurs pri r to administration,

an advantage is achieved in that th liposome with the gaseous precursor is a more stable entity than a lip some which has been placed n the shelf with a gas encapsulated therein. Accordingly, greater shelf life is evident from the use of liposomes which encapsulate a pH-activated gaseous precursor. It has also been discovered that the use of ionophores allows liposomes entrapping pH-activated gaseous precursors to efficiently produce gas when exposed to a pH gradient. The resulting gas-containing liposomes are capable of being detected easily in vivo because of their lower density as compared to the surrounding bodily structures and organs.

In a second embodiment of the invention, a contrast agent for ultrasonic imaging is provided which comprises a liposome having encapsulated therein a photo- activated gaseous precursor. As used herein, the phrase "phot - activated gaseous precursor" denotes a light sensitive chemical which forms a gas after exposure to such light. This concept is illustrated in Figure 1B. Suitable photosensitive compounds include diazonium compounds which decompose to form nitrogen gas after exposure to ultraviolet light. Another suitable compound is aminomalonate. As one skilled in the art would recognize, other gaseous precursors may be chosen which form gas after exposure to light. Depending upon the application, exposure to such light may be necessary prior to in vivo administration, or in some instances can occur subsequent to in vivo administration. Even when exposure to the appropriate light occurs prior to administration, an advantage is achieved in that the liposome with the gaseous precursor is a more stable entity than a liposome which has been placed on the shelf with a gas encapsulated therein. Accordingly, greater shelf life is evident from the use of the liposome which encapsulates a photo-activated gaseous precursor. The resulting gas- c ntaining liposomes are capable of b ing detected easily in viv becaus of th ir l w r density as c mpar d to th surr unding bodily structur s and rgans.

In a third emb dim nt, th pr s nt invention is

directed to a contrast agent for ultras nic imaging which comprises a liposome having encapsulated therein a temperature-activated gase us precurs r. As used herein, the phrase "temperature-activated gaseous precursor" denotes a compound which forms a gas following a change in temperature. This concept is illustrated in Figure 1C. Suitable temperature-activated gaseous precursors are well known to those skilled in the art, and include, for example, methylactate, a compound which is in a liquid phase at ambi nt temperatures, but which forms a gas at physiological temperatures. As those skilled in the art would recognize, such compounds can be activated prior to administration or, as in the case of methylactate, can be activated upon injection into the patient. Even when exposure to the appropriate temperature occurs prior to administration, an advantage is achieved in that the liposome with the gaseous precursor is a more stable entity than a liposome which has been placed on the shelf with a gas encapsulated therein. Accordingly, greater shelf life is evident from the use of th liposome which encapsulates a temperature-activated gaseous precursor. The resulting gas-containing liposomes are capable of being detected easily in vivo because of their lower density as compared to the surrounding bodily structures and organs. In addition, as those skilled in the art would recognize, such temperature sensitive gas-forming liposomes can be used as indicators of in vivo temperature.

Liposomes encapsulating solid and liquid contrast enhancing agents are also encompassed within the subject invention. As used herein, the terms "solid contrast enhancing agent" and "liquid contrast enhancing agent", denotes solid particulate materials, and solubilized or liquid materials, respectively, which are echogenic on ultrasound. Suitable solid contrast enhancing agents will be readily apparent t tho skilled in th art nce armed with the present disclosure, and includ magnetit (Fe_3O_4), solid iodin particles such as particles formed from iodipamide ethyl ster, and particles formed by precipitating a water

insoluble derivative of the ionic iodinated contrast medium metricate. Suitable liquid contrast enhancing agents will be readily apparent to those skill d in the art, nce armed with the present disclosure, and include solubilized iodinated contrast agents. The latter is preferably used as an intravascular contrast agent for the purpose of visualizing flow, but is also highly effective for detecting tumors in the liver and spleen.

As those skilled in the art will recognize upon reading the present disclosure, some solid and liquid contrast enhancing agents can be formed in situ. In the case of magnetite, for example, iron salts can be encapsulated at low pH (e.g., pH 2) and the external pH of the outside solution raised. The iron oxides then precipitate within the vesicle forming magnetite. To facilitate the transport of hydroxide ion into the vesicle, an ionophore such as valinomycin is incorporated into the liposome membrane. This is similar to the situation shown in Figure 1D, except that in this instance, pH is being raised, rather than lowered.

The liposomes employed in the present invention can be prepared using any one of a variety of conventional liposome preparatory techniques. As will be readily apparent to those skilled in the art, such conventional techniques include sonication, chelate dialysis, homogenization, solvent infusion coupled with extrusion, freeze-thaw extrusion, microemulsification, as well as others. These techniques, as well as others, are discussed, for example, in U.S. Patent No. 4,728,578, U.K. Patent Application G.B. 2193095 A, U.S. Pat nt No. 4,728,575, U.S. Patent No. 4,737,323, International Application PCT/US85/01161, Mayer et al., Biochimica et Biophysica Acta, Vol. 858, pp. 161-168 (1986), Hope et al., Biochimica et Biophysica Acta, Vol. 812, pp. 55-65 (1985), U.S. Patent No. 4,533,254, Mahew et al., Methods In Enzymology, V l. 149, pp. 64-77 (1987), Mahew et al., Biochimica et Biophysica Acta, V l. 75, pp. 169-174 (1984), and Ch ng t al., Investigative Radiology, Vol. 22, pp. 47-55 (1987), and U.S. S rial No. 428,339, filed Oct. 27, 1989.

The disclosures of each of the for going patents, publications and patent applications are incorporated by reference herein, in their entirety. As a preferred technique, a solvent free system similar to that described in International Application PCT/US85/01161, or U.S. Serial No. 428,339, filed Oct. 27, 1989, is employed in preparing the liposome constructions. By following these procedures, one is able to prepare liposomes having encapsulated therein a gaseous precursor or a solid or liquid contrast enhancing agent.

The materials which may be utilized in preparing the liposomes of the present invention include any of the materials or combinations thereof known to those skilled in the art as suitable in liposome construction. The lipids used may be of either natural or synthetic origin. Such materials include, but are not limited to, lipids such as cholesterol, phosphatidylcholine, phosphatidylethanolamin , phosphatidylserine, phosphatidylglycerol, phosphatidicacid, phosphatidylinositol, lysolipids, fatty acids, sphingomyelin, glycosphingolipids, glucolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids, polymerizable lipids, and combinations thereof. As one skilled in the art will recognize, the liposomes may be synthesized in the absence or presence of incorporated glycolipid, complex carbohydrate, protein or synthetic polymer, using conventional procedures. The surface of a liposome may also be modified with a polymer, such as, for example, with polyethylene glycol (PEG), using procedures readily apparent to those skilled in the art. This is illustrated in Figure 2. Any species of lipid may be used, with the sole proviso that the lipid or combination of lipids and associated materials incorporated within the lipid matrix should form a bilayer phase under physiologically relevant conditi ns. As on skilled in th art will recognize, th comp sition f th liposomes may b altered t modulat the biodistribution and clearance prop rties of the resulting liposomes.

To incorporate ionophores into the liposome membrane, the ionophores, which are lipophilic, are simply added to th lipid mixture, and the liposomes are prepared in the usual fashion.

In addition, the size of the vesicles can be adjusted by a variety of procedures including filtration, sonication, homogenization and similar methods to modulate liposomal biodistribution and clearance. To increase internal aqueous trap volume, the vesicles can be subjected to repeated cycles of freezing and thawing.

The liposomes of the invention may be of varying sizes, but preferably have a mean outer diameter between about 30 nanometers and about 10 microns. As is known to those skilled in the art, vesicle size influences biodistribution and, therefore, different size vesicles are selected for various purposes. For intravascular use, for example, vesicle size is generally no larger than about 2 microns, and generally no smaller than about 30 nanometers, in mean outer diameter. In the size range of 2-3 microns, the vesicles are by their nature multilamellar. Within this range, to maximize echogenicity with a liposomal contrast agent which has a short intravascular half life, larger vesicles are selected, e.g., about 1 to about 2 microns in mean outer diameter. For sustained blood pool imaging such as for perfusion, smaller vesicles are used, e.g., between about 100 nanometers and several hundred nanometers in mean outer diameter. To provide ultrasound enhancement of organs such as the liver and to allow differentiation of tumor from normal tissue smaller vesicles between about 30 nm and about 100 nm in mean outer diameter which will cross the capillary fenestrations into the liver and increase the uptake by liver may be employed. For imaging of body cavities and non-vascular injection, larger vesicles, e.g., between about 2 and about 10 micron m an utside diam ter may be empl yed to maximize chogenicity f entrapped air.

The lipids employ d in th preparations are s lected t optimize the particular diagnostic us , minimize toxicity

and maximize shelf-life of the product. Neutral vesicles composed of phosphatidylcholine and ch lesterol function quite well as intravascular contrast agents to entrap gas, magnetite, solid iodine particles and solubilized iodinated contrast agents. To improve uptake by cells such as th reticuloendothelial system (RES), a negatively charged lipid such as phosphatidylglycerol, phosphatidylserine or similar materials is added. To prolong the blood pool half life, highly saturated lipids which are in the gel state at physiological temperature such as dipalmitoylphosphatidyl-choline are used. For even greater vesicle stability and prolongation of blood pool half-life the liposome can be polymerized using polymerizable lipids, or the surface of the vesicle can be coated with polymers such as polyethylene glycol so as to protect the surface of the vesicle from serum proteins, or gangliosides such as GM1 can be incorporated within the lipid matrix.

The pH-activated gaseous precursor, the photo-activated gaseous precursor, the temperature-activated gaseous precursor, and/or the solid or liquid contrast enhancing ag nt can be incorporated into the liposome by being added to the medium in which the liposome is being formed, in accordance with conventional protocol.

The liposomes of the present invention are useful in ultrasound imaging.

In a still further embodiment, the present invention comprises novel devices and methods for encapsulating a gas within the internal space of the liposome. The liposomes thus produced are also useful in ultrasound imaging.

In general terms, in using the device and carrying out the method of the invention, liposomes are added to a vessel, and the vessel is then pressurized with gas. Under pressure, the gas goes into solution and passes across the liposome membranes. When the pr ssur is releas d, gas bubbles f rm within the liposomes. Figur 3 illustrates the general pressurization and depressurization phenomenons of the inv ntion.

In using the pressurization devices and carrying out the pressurization processes of the invention, it is preferable t utilize a liposome that does not c ntain a sterol as part of the membrane. The presence of sterols such as cholesterol in the liposome membrane, particularly if they are present in significant quantities, i.e., greater than about 5% of the membrane by volume, tends to substantially inhibit the flow of certain solubilized gases, such as carbon dioxide, across the liposome membrane. Also, if the membran is comprised largely of saturated lipids, that is greater than about 80% saturated lipids, the flow of certain solubilized gases, such as that of carbon dioxide, is also substantially inhibited.

The liposomes may or may not be pre-sized prior to being placed within the pressurization vessel. In the case of pre-sizing this may be achieved by a variety of means including, but not limited to, sonication, gel-filtration, filtration through polystyrene or polycarbonate filters or other filters of suitable material, French press or micro-emulsification methods.

There is shown is Figure 4 an apparatus for synthesizing liposomes having encapsulated therein a gas. The apparatus is, in essence, a modified soda seltzer bottle. The apparatus is utilized by placing a liquid media, such as a phosphate buffered saline solution which contains liposomes, into a vessel (1). Typically, the liposomes will be comprised of egg phosphatidylcholine, although as described above, other lipids can be employed in the preparation of the liposomes. A cap (10) is then threaded onto the vessel opening (11), providing a pressure tight seal. The vessel is pressurized by fitting a cartridge (9) containing a gas or a combinati n of gases, such as carbon dioxide, into an inlet port (8). The vessel may be constructed of any suitable material, such as glass r acrylic, and may be disp sabl , if desired. The cartridg discharges its c ntents into the upper nd (6) of a tube (3), preferably a polyethylene tube, fitted into th vessel (1). The gas flows thr ugh the tub and exits at th

lower end (4) of the tube. Th gas then bubbles upward thr ugh the liquid media so that at least a p rtion of the gas dissolves in the liquid media. Generally, the pressure of the gas in this and other pressurization devices and methods disclosed herein, is between about 2 and about 400 psi. In the preferred embodiment, the vessel is pressurized to between the 50-120 psi range. Within this range, generally higher pressures are preferred for certain gases, such as nitrogen, and gradually lower pressures required for others, such as carbon dioxide. If necessary, additional gas cartridges may be used. A pressure gauge (29) indicates the pressure in the vessel.

The liposome membranes are permeable to the pressurized gas. Thus, as the gas bubbles through the liquid media, a portion of the dissolved gas is encapsulated within the internal aqueous environment of the liposomes. To enhance the dissolving of the gas into the liquid, it is desirable to promote mixing of the gas and the liquid, and bubbling the gas through the liquid assists in this. In the preferred embodiment, this mixing is further enhanced by providing the vessel with a convex shaped bottom (2) projecting into the vessel. The lower end of the tube discharges the gas near the most inward point (5) on the convex shaped bottom.

After the gas has been introduced into the vessel (1), the vessel is depressurized by ejecting the liquid therefrom. Ejection is accomplished by actuating a discharge lever (12), in the cap (10). Actuation of the discharge lever opens an outlet port (7) so that the gas pressure forces the liquid to enter the tube (3) at its lower end (4), flow up the tube and out of the vessel through the discharge port. Forcing the liquid through the tube promotes further mixing of the gas and liquid. Upon depressurization, the dissolved gas encapsulated by the liposomes comes out of solution and forms bubbles within the lip somes, th r by forming liposomes having encapsulated therein a gas.

Alternatively, the method described abov could be practiced using the apparatus shown in Figure 5. If d sired,

th liposomes may be pre-sized by injecting them from a syringe (33) through one or more filter(s) (30), through inlet/outlet port (35) and valve (39), and then through tube (38) into vessel (34). Alternatively, the liposomes are simply placed in a vessel (34). Vessel (34) is equipped with valves (39), inlet/outlet port (35), inlet/outlet port (36) and tube (38). The vessel (34) is constructed so that it can be pressurized with a gas or combination of gases such as carbon dioxide, oxygen, nitrogen, xenon, argon, neon and helium by means of a valve or inlet port (36) and external pressure source (37) which can be a gas line, tank or disposable cartridge. The valve(s) (39) may be constructed so as to be able to vent excess pressure without dispensing the liposomes. The vessel may be constructed of any suitabl material, such as glass or acrylic, and may be disposable, if desired.

In use, the vessel (34) is first loaded with a liposomal containing solution, using if desired, syringe (33), with or without filter (30). The vessel is then pressurized with gas using external pressure source (37) which passes gas through inlet port (36) through valve (39) and tube (38) into vessel (34). Under pressure, the gas goes into solution and passes across the liposome membranes. When the pressure is released, gas bubbles form within the liposomes. The pressurized vessel (34) has an inlet/outlet port (35) to which one or more filters (30) may be attached. In Figure 5, the use of Luer lock fittings (31) and (32) is illustrated as an example of a means for connecting the pressure vessel (34), filters (30) and syringe (33). However, any suitable means of coupling the devices may be employed. The pore size of suitable filters may vary widely between about 0.015 micron and about 10 microns. More than one filter, if desired, may be employed in serial connection. The function of the filter(s) (30) is threefold: to promote decavitation of any bubbles formed external to the liposomes after preparation of the gas-encapsulating liposomes; to promote sizing of the liposomes either before or after gas-encapsulation; and to

rem ve non-liposomal solids from the suspension. The filt r(s) (30) may in turn be c nnected t a syring (33). When the v ssel is filled with liposomes, they may be directed from the syringe (33) through the filter(s) (30) and through the inlet/outlet port (35) and valve (39) into pressure vessel (34). In addition, when the contents of vessel (34) are released by means of the outlet valve (35) of the vessel, the output stream may be directed through the filters (30) into the syringe (33). If desired, the vessel (34) may be directly loaded and unloaded without passing through inlet/outlet port (35) and valve (39), filter (30) and/or syringe (33). The advantage of this procedure is that the device can be pre-packaged as a stand-alone, sterile unit ready for use. It should also be noted that it is not necessary that either the filters or syringe be used as described, e.g., the output stream of the device may be directed into a separate container prior to being taken up, for example, into a syringe for injection.

The method discussed above could also be practiced using the apparatus shown in Figure 6, wherein the gas enters vessel (13) in which a liquid media containing liposomes has been placed, through inlet port (14), flows through tube (15) and discharges into the bottom of the vessel. The vessel may be constructed of any suitable material, such as glass or acrylic, and may be disposable, if desired. From the bottom of the vessel, the gas bubbles upward through the liquid. Depressurization is accomplished by opening a valve, not shown, on the outlet port (16), thereby ejecting the liquid from the bottom of the vessel through tube (17).

Referring to Figure 7, the apparatus required to practice such a method need only be a simple vessel (27) with a port (28) for introducing the liposomes and the pressurized gas and discharging the same. The vessel may be constructed f any suitable mat rial, such as glass r acrylic, and may be disp sabl , if desir d.

Th invent rs have disc v red that with s me gases, such as with carbon di xid , it is h lpful t bubble the gas

through the liquid in rder to dissolve the gas. Also, with some gases, such as with nitr gen, it is helpful t co l the liquid to approximately the 1-4°C range. In carrying ut the pressurization, pressures between about 2 and about 400 psi, preferably between about 30 and about 100 psi should be employed. The inventors have also discovered that it is preferable that the depressurization occur quickly over several seconds or less.

It should be noted that the method described above is particularly adapted for use with liposomes having membranes which are relatively permeable to the gas. However, the inventors have found that by subjecting the liposome-containing liquid media to high frequency sound waves, as discussed below, even relatively impermeable membrane compositions can be easily utilized. As a general rule and as noted above, membranes composed of significant amounts of sterols or composed largely of saturated lipids are relatively impermeable to certain gases such as carbon dioxide, however, egg phosphatidyl choline, for example, is highly permeable.

There is shown in Figure 8 an apparatus for synthesizing liposomes containing gas which uses sonicati n. The apparatus is utilized by placing a liquid media containing liposomes, in a vessel (18). A high frequency sound wave generator (25), which may be a Sonicator (cr), available from Heat Systems-Ultrasonics, Inc., is attached to the vessel using mating threads (24) formed in the inlet to the vessel and the outside of the generator. The vessel is then pressurized using a gas introduced through gas port (22). In the preferred embodiment, the vessel is pressurized to betw en about 30 and about 120 psi using a gas, such as carbon dioxide, cooled into approximately the 1-4°C range, ther by promoting the dissolving of the gas into the liquid media. The vessel is jacketed by a chamber (19), through which a liquid or gas ous c olant (26) circulates via inlet and utlet p rts (20), (21) resp ctively, so as to maintain the t mp ratur of the liquid preferably between ab ut 1°C and about 4°C rang during the s nication pr cess d scrib d below.

The sound wave generator (25) transforms electrical energy into mechanical energy, at a frequency of approximately 20 kHz and an amplitude approximately in the range of 30-120 μm, by oscillating piezoelectric crystals. These oscillations are transmitted and focused into the liquid through a horn (23) which extends into the vessel (18), causing high frequency sound waves to propagate through the liquid. The sound waves cause cavitation in the liquid, i.e., the high frequency formation and collapse of microscopic bubbles. Th process of inducing cavitation by high frequency sound waves is referred to as sonication. The cavitation induces a shearing and tearing action in the liquid, causing the large multilamellar liposomes to tear and reform into smaller oligolamellar liposomes and eventually, depending on the duration and intensity of the sonication, unilamellar liposomes. As the liposomes break up and reform, they encapsulate the dissolved gas within their internal aqueous cores.

Following sonication, the vessel is depressurized and the encapsulated gas forms bubbles, thereby transforming the liposomes into gas containing liposomes as before. However, since the gas was introduced into the liposomes during their break-up caused by the sonication, liposomes having relatively impermeable membranes can be used. By using such membranes, an added advantage in stability is achieved, that is, the gas bubbles do not diffuse back across the membrane as readily as with other methods and membranes. Thus, the sonication process allows the formation of more stable gas containing liposomes. The inventors have found that generally cholesterol-based liposomal membranes are relatively impermeable.

Other techniques in addition to sonication can be used to synthesize liposom s having encapsulated therein a gas, including microemulsification, extrusion, micro-fluidizati n, h mogenizati n and the like, the requirement being that th synthetic process be c nducted under

pressurization, preferably at low temperatures.

In each of the foregoing methods for preparing gas-containing liposom s, the liposome preparation is preferably st red under pressure with the gas in solution or the gas pressurization and depressurization process is carried out at or about the time of use.

The liposomes of the present invention and those produced by the apparatus and method of the invention are useful in imaging a patient using ultrasound. The present invention is useful in imaging a patient generally, and/or in specifically diagnosing the presence of diseased tissue in a patient. The patient can be any type of mammal, but most preferably is a human. The method of the invention is particularly useful in diagnosing the vasculature, that is, the arterial system, the venous system and the heart. It is also particularly useful in providing images of the patient's liver, spleen or kidney.

The imaging process of the present invention may be carried out by administering a liposome of the invention, that is a liposome selected from the group consisting of an ionophore-containing liposome having encapsulated therein a pH-activated gaseous precursor, a liposome having encapsulated therein a photo-activated gaseous precursor, a liposome having encapsulated therein a temperature-activated gaseous precursor, and/or a liposome having encapsulated therein a solid or liquid contrast enhancing agent, to a patient, and then scanning the patient using ultrasound imaging to obtain physical images of an internal region of a patient and/or of any diseased tissue in that region. By region of a patient, it is meant the whole patient or a particular area or portion of the patient.

Any of the various types of ultrasound imaging devices can be employed in the practice of the invention, the particular typ r model of the d vic not b ing critical to the method of the inv nti n.

F r intravascular use the contrast agent is generally injected intravenously, but may be injected intra-

arterially also. As injections are perf rmed, ultrasonic images are obtained with an ultras und scanner. In the case of intravascular injection, the liposomal contrast agents generally have a mean outer diameter of smaller than about 2 to about 3 microns (small enough to pass through the pulmonary circulation). In other non-vascular applications, the liposomal contrast agent may be injected directly into the area to be scanned, into sites such as sinus tracts or the uterine cavity, for example, to assess patency of the fallopian tubes. In cases of non-vascular injection, the liposomal contrast agent diameter is not constrained by the necessity of passing through the pulmonary microvasculature. Therefore larger liposomes can be used to maximize echogenicity.

In administering the liposomes of the present invention, dosage is typically initiated at lower levels and increased until the desired contrast enhancement in the patient is achieved. In carrying out the method of the invention, the liposomes can be used alone, in combination with one another, or in combination with other diagnostic and/or therapeutic agents. Preferable routes of administration will be readily apparent to those skilled in the art. As those skilled in the art will recognize, such parameters as dosage and preferable administration routes will vary depending upon the age, weight and mammal to be diagnosed, the particular type of liposome to be employed, and most importantly, the particular area of the patient to be scanned.

The following Examples are merely illustrative of the present invention and should not be considered as limiting the scope of the invention in any way. These examples and equivalents thereof will become more apparent to those versed in the art in light of the present disclosure, and the accompanying Claims.

Examples

Example 1

Egg phosphatidylcholine, 1 gram, was suspended in 100 cc of physiological saline at room temperature to form a dispersion of multilamellar liposome vesicles. The liposomes were then placed in the vessel of Figure 5. The outlet valve on the vessel was then sealed and the system was pressurized with between 30 to 50 psi CO_2 gas. The suspension is then emptied into a flask and the non-encapsulated CO_2 gas was allowed to escape. CO_2 gas entrapped within the vesicles remained entrapped. The gas filled vesicles surprisingly did not float, but were distributed evenly in solution. The resultant gas filled liposomes were found to be intensely echogenic on ultrasound.

Example 2

Vesicles were also formed as described in Example 1, except that vesicle formation was carried out in the presence of bicarbonate and the ionophore A23187 resulting in bicarbonate encapsulated liposomes contacting that ionophore. Acid was added to the external aqueous phase in order to lower the pH within the vesicles. The bicarbonate entrapped within the vesicles was found to form CO_2 gas and water.

Examples 3-18

A. Liposome Preparation

To prepare multilamellar vesicles (MLV's) pure egg phosphatidylcholine (EPC) obtained from Avanti Polar Lipids (Birmingham, Alabama) was suspended in phosphate buffered saline (PBS) and swirled by hand. In other cases vesicles of defined size were prepared by a process of extrusion with or without a preceding freeze-thaw process. Two different lipid mixtures were tested, either pure EPC or a mixture of 80 mole percent EPC with 20 mole percent cholesterol. Typically, for the EPC/cholesterol vesicles 3.6 mmol (2.83 g) of EPC and 1.2 mmol (0.48 g) of cholesterol were dissolved together, in a minimum v lume f chl r form, in a 250-ml round-bottom flask. The chloroform was rem ved by rotary evaporation und r r duced pressur to leav a thin film on th walls of th flask; the contents were th n h ld und r reduced pressure (less than 0.1

mm Hg) for at 1 ast 2 hours to r move residual solvent. For mixtur s of pur EPC the step f suspension in chlor form was omitted. For both pure EPC and the dried film of EPC/cholesterol, the lipid was dispersed by vigorous mixing in 20 cc of neutral pH phosphate buffered saline (PBS).

Vesicles prepared both with and without freeze-thaw were synthesized. For vesicles subjected to freez - thaw the multilamellar vesicles formed upon dispersion were transferred to cryovials and then quench frozen in liquid nitrogen. The cryovials were then placed in warm water until the lipid suspension had completely thawed. This cycle of freezing and thawing was repeated four more times. Both the vesicles subjected to freeze-thaw and those which were n t freeze-thawed were then sized by ten passes under nitrogen pressure (approx. 100 psi) through two stacked 2 micron filters (Nucleopore, Pleasanton, Calif) using the extruder device (Lipex Biomembranes, Vancouver, British Columbia, Canada). A portion of this sized preparation was then passed ten times through two stacked 0.4 micron filters, a portion of this was then passed ten additional times through 0.2 micron filters, and finally a portion of this was passed ten times through 0.030 micron filters. The above was conducted for the EPC/cholesterol vesicles but filter sizes of only 0.4 and 0.2 microns were tested for the vesicles composed of pure EPC. The sizes of the resultant vesicles were determined by quasi elastic light scattering with a Nicomp (Goleta, Calif) Model 270 particle sizer operating at 634.2 nm by standard cumulants analysis.

B. Pressurization

Pressurization of the liposome solutions and controls of PBS was accomplished with a soda seltzer bottle using carbon dioxide cartridges (Figure 4). In all experiments for dilution of liposomes and as controls only d gass d s luti ns f PBS wer used. All liquids were degassed immediately prior to us . Degassification of soluti ns was acc mplished by reducing pressur under vacuum. In pressurizati n f all s luti ns 50 cc f liquid was poured

into the seltzer bottle and the system then sealed. Pressurization was then accomplished by engaging the CO_2 cartridge with the b ttle. After 1 minute the pressure was released from the bottle and the solution poured from the bottle. The seltzer bottle was equipped with a pressure gauge and the pressure was measured during each experiment. Solutions which were exposed to gassification included various dilutions of the different sizes of liposomes and pure PBS.

C. Ultrasound Imaging

Ultrasound Imaging was performed with an Acuson 128 scanner (Milpitas, CA.) using a 7.5 megahertz linear array transducer. Post-processing function was linear with pre-processing set at 0 and persistence at 2. Phantom soluti ns (controls and liposomal agents) at variable concentrations and constant volumes and depth were scanned at 30 to 60 dB gain settings within thin plastic containers. Multifocal zones with a decreased frame rate were used for most images. For quantitative measurements a 1 cm circle was positioned on the images at a position 2 cm below the transducer and the number of reflections within the circle was counted. At the time of measurement of acoustic reflections the ultrasonographer was blinded to information as to which contrast agent or control was being studied.

D. Results

The sizes of the resulting vesicles and their echogenicity on ultrasound are shown in Tables I and II. The size of the vesicles is controlled by the filters used in the extrusion process. For the MLV's prepared without extrusion the size range is quite variable. For those vesicles which underwent the extrusion process the size range is comparatively narrow.

In the ultrasound imaging experiments of the phantoms c ntaining the diff r nt contrast agents large reflections wer s en at 30 dB f r the first minute or two after the ultras und c ntrast agents wer pressurized and then p ured into the plastic phant m dishes. These large

reflections decayed quickly however and were not visible after several minutes. The reflections which persisted after the first 2 to 3 minutes were of much finer size and more regular in appearance.

The number of reflections counted within a 1 cm diameter circle over time for the different solutions is shown in Tables I and II. The 0.4 micron vesicles composed of pure EPC synthesized by extrusion but without freeze-thaw had the greatest echogenicity after exposure to pressurization and echogenicity was sustained for 2 hours after the pressurization process. By comparison the phantom containing a similar concentration of the same vesicles without exposure to pressurization had no internal echoes at all at 30 minutes after they were poured into the phantom. MLV's synthesized of pure EPC had the next highest echogenicity. All of the vesicles which contained cholesterol had lower echogenicity than the vesicles composed of pure EPC. The control solution of PBS exposed to pressurization but not containing liposomes had echoes during the first minute or two but these echoes decayed rapidly to zero after several minutes (Table II).

The greatest echogenicity was seen in 0.4 micron vesicles composed of pure EPC which were extruded but not freeze-thawed. Vesicles of the same size which were freeze-thawed had less echogenicity. Vesicles of this size subjected to freeze-thaw will be unilamellar whereas those not subjected to freeze-thaw of this size will be oligolamellar.

Table I*

Number of Reflections at 60 dB

Example		Time in minutes 5	10	20	30	45	60	120
3	(0.4 μ, no FT)	-	-	-	95	89	116	109
4	(0.4 μ + FT)	-	-	-	52	69	-	-
5	(0.2 μ + FT)	-	-	-	26	52	-	-
6	(MLV's)	-	-	58	72	-	-	-
7	(Ex. 6 at 94 psi)	-	60	69	-	-	-	-
8	(MLV's, no gas)	84	32	10	-	-	-	-
9	(Ex. 3, no gas)	18	-	-	0	-	-	-
10	(PBS)	0	-	-	-	-	-	-
11	(PBS + gas)	12	10	3	-	-	-	-

*Data above from counting number of reflections within 1 cm diameter circle positioned 2 cm from transducer on images obtained by scanning 400 cc solutions of ultrasound contrast agents with 7.5 mHz linear array transducer. The liposomes in Examples 3 through 6 were pressurized with 52 to 54 psi CO_2 gas. The liposomes in Examples 3, 4 and 5 were extruded 10 times through filters as specified, in Examples 4 and 5, these liposomes were then exposed to 5 cycles of freeze-thaw, and in Example 3, these liposomes were not freeze-thawed. The liposomes in Example 6 involved multilamellar vesicles (MLV's) prepared by simple mixing of egg phosphatidylcholine (EPC). The liposomes in Example 7 were exposed to higher pressur of 94 psi. The liposomes in Example 8 and 9 are control samples of vesicles, with those in Example 8 involving MLV's and those in Example 9 being 0.4 μ vesicles (no freeze-thaw) without exposure to gas. In Example 10, the sample is phosphate buffered normal saline (PBS) without exposure to gas. In all vesicl pr parati ns final lipid concentration is 1.25 micr m les/ml. Th n tati n (-) indicates that the numb r of reflecti ns was n t measur d.

Table II*

Number of Reflecti ns at 60 dB

Example		Time in minutes 1	2	3	10	15	20	30
12	(0.2 μ)	22	30	33	-	-	-	27
13	(MLV's)	40	27	34	-	-	-	48
14	(2.0 μ EPC/Chol.)	-	18	-	-	15	15	-
15	(MLV's 50 nm EPC/Chol.)	12	23	13	-	-	16	15
16	(0.4 μ EPC/ Chol.)	15	22	-	-	11	-	15
17	(PBS + gas)	15	7	6	10	3	0	0
18	(PBS, no gas)	23	25	4	-	-	0	-

*Lipid concentration in the above is 0.225 micromoles of lipid per ml. The liposomes in Examples 12 and 13 are pure EPC, the liposomes in Example 13 are MLV's as in Table I, and the liposomes in Example 12 are a dilute version of those employed in Example 5 from Table I. Examples 12, 14, 15 and 16 wer produced by extrusion through filter pore sizes as specified and Examples 14, 15 and 16 contain 80% EPC/20% Cholesterol.

Having described the invention above, it will be obvious to one skilled in the art that various parameters such as liposome size and membrane composition are selected to achieve the desired effect in terms of biodistribution and imaging.

Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in th art fr m the foreg ing description. Such m dificati ns ar als int nded to fall within th scope of the appended claims.

CLAIMS

What is claimed is:

1. A contrast agent for ultrasonic imaging which comprises an ionophore-containing liposome having encapsulated therein a pH-activated gaseous precursor.

2. A contrast agent according to Claim 1 wherein said pH-activated gaseous precursor is selected from the group consisting of metal carbonate and metal bicarbonate salts.

3. A contrast agent according to Claim 2 wherein said pH-activated gaseous precursor is sodium bicarbonate.

4. A contrast agent according to Claim 1 wherein said ionophore in said ionophore-containing liposome is selected from the group consisting of carbonylcyanide, p-trifluoromethoxyphenylhydrazone, carbonylcyanide M-chlorophenylhydrazone, carbonylcyanide phenylhydrazine, tetrachloro-2-trifluoromethyl benzimidazole, 5,6-dichloro-2-trifluoromethyl benzimidazole, Uncoupler 1799, gramicidin, alamethicin, filipin, etruscomycin, nystatin, pimaricin, amphotericin, valinomycin, enniatin, beauvericin, monomycin, nonactin, monactin, dinactin, trinactin, tetranactin, antamanide, nigericin, monensin, salinomycin, narisin, mutalomycin, carriomycin, dianemycin, septamycin, A-204 A, X-206, X-537 A, A-23187 and dicyclohexyl-18-crown-6.

5. A contrast agent for ultrasonic imaging which comprises a liposome having encapsulated therein a photo-activated gaseous precursor.

6. A contrast agent according to Claim 5 wherein said photo-activated precursor is a diazonium compound.

7. A contrast agent for ultrasonic imaging which

comprises a liposome having ncapsulated therein a temperature-activated gase us precursor.

8. A c ntrast agent acc rding to Claim 7 wherein said temperature-activated gaseous precursor is methyllactate.

9. A contrast agent for ultrasonic imaging which comprises a liposome having encapsulated therein a solid or liquid contrast enhancing agent.

10. A contrast agent of Claim 9 wherein said solid contrast enhancing agent is selected from the group consisting of magnetite and iodipamide ethyl ester.

11. A method of providing an image of an internal region of a patient comprising:

(a) administering to the patient a contrast agent selected from the group consisting of (i) an ionophore-containing liposome having encapsulated therein a pH-activated gaseous precursor, (ii) a liposome having encapsulated therein a photo-activated gaseous precursor, (iii) a liposome having encapsulated therein a temperature-activated gaseous precursor, and (iv) a liposome having encapsulated therein a solid or liquid contrast enhancing agent; and

(b) scanning the patient using ultrasonic imaging to obtain visible images of the region.

12. The method according to Claim 11 wherein said liposomes are administered intravascularly, and the vesicles have a mean outer diameter of between about 30 nanometers and about 2 microns.

13. The method acc rding t Claim 12 wh r in the patient is scanned in th area of th pati nt's heart.

14. The method acc rding to Claim 12 wherein the

patient is scanned in the area of the patient's arterial system.

15. The method according to Claim 12 wherein the patient is scanned in the area of the patient's venous system.

16. The method according to Claim 12 wherein the patient is scanned in the area of the patient's liver, spleen and kidney.

17. The method according to Claim 11 wherein the liposomes are administered other than intravascularly and have a mean outer diameter between about 2 microns and about 10 microns.

18. The method of Claim 11 wherein the liposomes are comprised of lipids of either natural or synthetic origin selected from the group consisting of fatty acids, lysolipids, dipalmitoylphosphatidylcholine, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, phosphatidylinositol, sphingomyelin, lysolipids, cholesterol, cholesterol hemisuccinate, glycosphingolipids, glycolipids, glucolipids, sulphatides, and tocopherol hemisuccinate.

19. A method for diagnosing the presence of diseased tissue in a patient comprising:

(a) administering to the patient a contrast agent selected from the group consisting of (i) an ionophore-containing liposome having encapsulated therein a pH-activated gaseous precursor, (ii) a liposome having encapsulated therein a photo-activated gaseous precursor, (iii) a liposome having encapsulated therein a temperature-activated gaseous precursor, and (iv) a liposome having encapsulated therein a

solid or liquid contrast enhancing agent; and

(b) scanning the patient using ultrasonic imaging to obtain visible images of any diseased tissue in the patient.

20. A method of synthesizing a liposome having encapsulated therein a gas comprising the steps of:

(a) placing a liquid media containing liposomes in a vessel,

(b) pressurizing said vessel with a cooled gas, thereby dissolving a first portion of said gas into said liquid media, so that a portion of said dissolved gas enters said liposomes, and

(c) rapidly depressurizing said vessel by releasing a second portion of said gas from said vessel, thereby causing said dissolved gas having entered said liposomes to form bubbles within said liposomes.

21. The method according to Claim 20 wherein said gas is selected from the group consisting of nitrogen, oxygen, carbon dioxide, xenon, argon, neon and helium.

22. The method according to Claim 20 wherein said liquid media is a phosphate buffered saline solution.

23. The method according to Claim 20 wherein said liposomes are comprised of egg phosphatidylcholine.

24. The method according to Claim 20 wherein th step of pressurizing said vessel comprises the step of pressurizing said vessel to a pressure between about 50 and about 120 psi.

25. The method according to Claim 20 wherein the temperature of said cooled gas prior to said pressurization

of said vessel is between about 1 to about 4°C.

26. The method according to Claim 29 wherein the step f depressurizing said vessel is performed in less than about 5 seconds.

27. A method of synthesizing a liposome having encapsulated therein a gas comprising the steps of:

(a) placing a liquid media containing liposomes in a vessel,

(b) pressurizing said vessel with a gas,

(c) bubbling said gas through said liquid media, thereby dissolving a portion of said gas in said liquid media, so that a portion of said dissolved gas enters said liposomes, and

(d) depressurizing said vessel.

28. The method according to Claim 27 wherein the step of depressurizing said vessel further comprises the step of rapidly releasing a portion of said liquid media from said vessel.

29. The method according to Claim 28 wherein: (a) said vessel is approximately cylindrically shaped, said vessel having an inlet port, an outlet port, and a bottom, a tube disposed in said vessel, said tube having first and second ends, said first end of said tube disposed in said bottom of said vessel, said second end of said tube in flow communication with said inlet port, and

(b) the step of pressurizing said vessel and bubbling said gas comprises the steps of introducing said gas into said inlet p rt under pressure and allowing said gas to flow thr ugh said tube, said gas discharging from said tube

at said first nd f said tube.

30. The method according to Claim 29 wherein: (a) said second end of said tub is in flow communication with said outlet port, and

(b) the step of depressurizing said vessel comprises the step of allowing said pressurizing gas to force said liquid to flow through said tube and out said outlet port.

31. The method according to Claim 29 wherein said bottom of said vessel curves convexly inward, said first end of said tube being closest to the most inward point on said convex bottom.

32. The method according to Claim 27 wherein said gas is selected from a group consisting of nitrogen, oxygen, carbon dioxide, xenon, argon, neon and helium.

33. The method according to Claim 27 wherein the step of pressurizing said vessel comprises the step f pressurizing said vessel to a pressure in approximately the 50-120 psi range.

34. The method according to Claim 27 wherein said liquid media is a phosphate buffered saline solution.

35. The method according to Claim 27 wherein said liposomes are comprised of egg phosphatidylcholine.

36. A method for synthesizing a liposome having encapsulated therein a gas comprising the steps of:

(a) placing a liquid media containing liposomes in a vessel,

(b) pressurizing said vess l with a gas,

(c) cavitating said liquid m dia,

(d) cooling said liquid media during said cavitation, and

(e) depressurizing said vessel.

37. The method according to Claim 36 wherein the step of cavitating said liquid media comprises the step of generating high frequency sound waves in said liquid media.

38. The method according to Claim 37 wherein th step of generating high frequency sound waves comprises the step of generating waves at a frequency of about 20 kHz and an amplitude about in the range of about 30-120 μm.

39. The method according to Claim 37 wherein the step of generating high frequency sound waves in said liquid media comprises the step of sonicating said liquid media.

40. The method according to Claim 38 wherein the step of pressurizing said vessel comprises the step of pressurizing said vessel to a pressure in the range of about 50 to about 120 psi using carbon dioxide cooled to about 1 to about 4°C range.

41. The method according to Claim 39 wherein the step of cooling said liquid media comprises the step of circulating a cooling fluid over the exterior of said vessel.

42. The method according to Claim 40 wherein the step of cooling said liquid media comprises the step of maintaining the temperature of said liquid media in a range of about 1°C to about 4°C.

43. The method according to Claim 40 wherein said lip som s c mprise cholest r l mixed with egg phosphatidylcholin .

44. The meth d acc rding t Claim 40 wherein said

liposomes comprise cholesterol mixed with saturated phospholipids.

45. A method for synthesizing a liposome having encapsulated therein a gas comprising contacting liposomes in an aqueous medium with gas under elevated pressures.

46. A method according to Claim 45 wherein said pressure is between about 50 and about 120 psi.

47. A method according to Claim 45 further comprising contacting said liposomes with gas under decreased temperature.

48. A method according to Claim 47 wherein said decreased temperature is between about 1 and about 4°C.

49. A method according to Claim 45 wherein said gas is selected from the group consisting of nitrogen, oxygen, carbon dioxide, xenon, argon, neon and helium.

50. A method according to Claim 45 wherein said liposomes comprise egg phosphatidylcholine.

Fig. IA

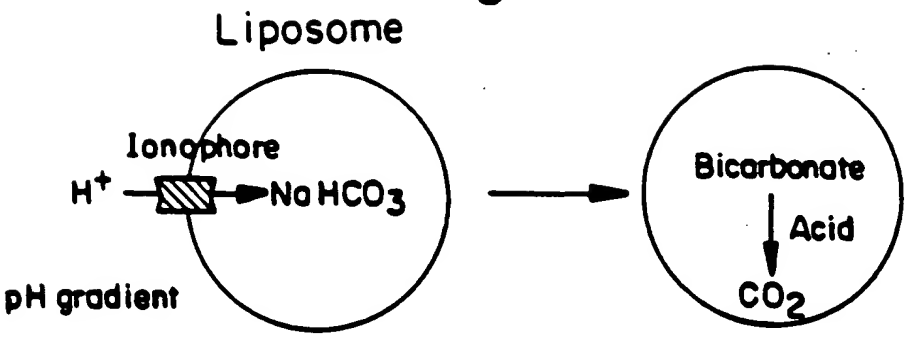


Fig. IB

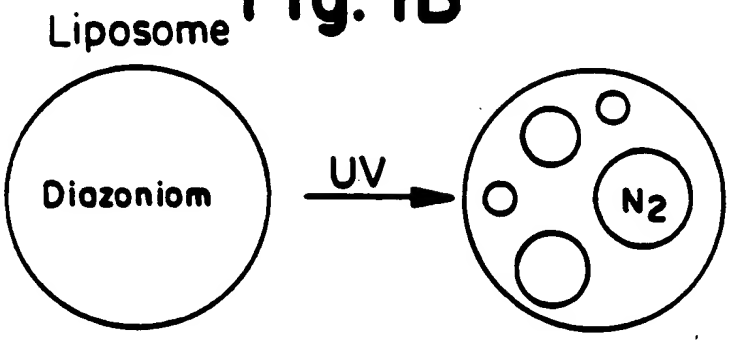
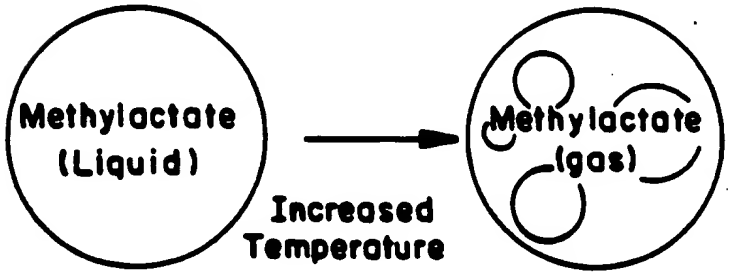


Fig. IC



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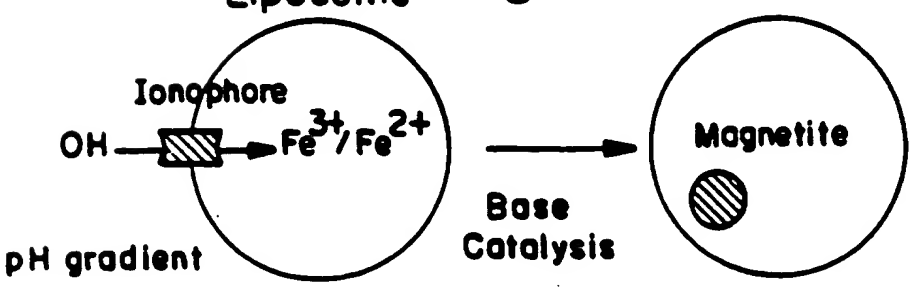


Fig. 2

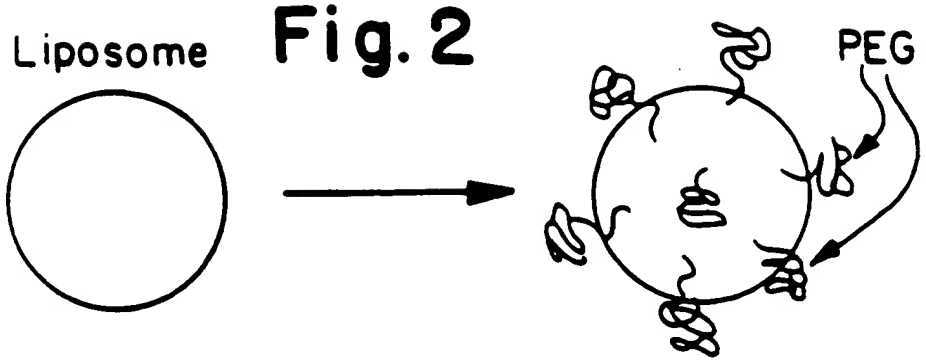
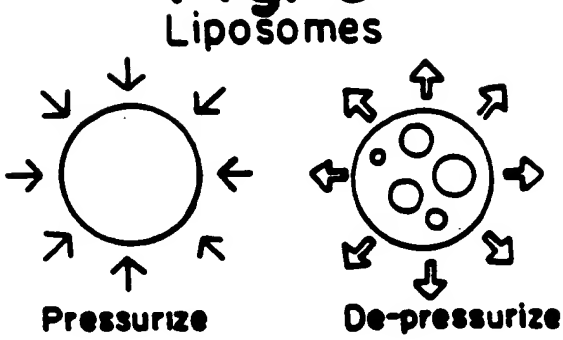


Fig. 3



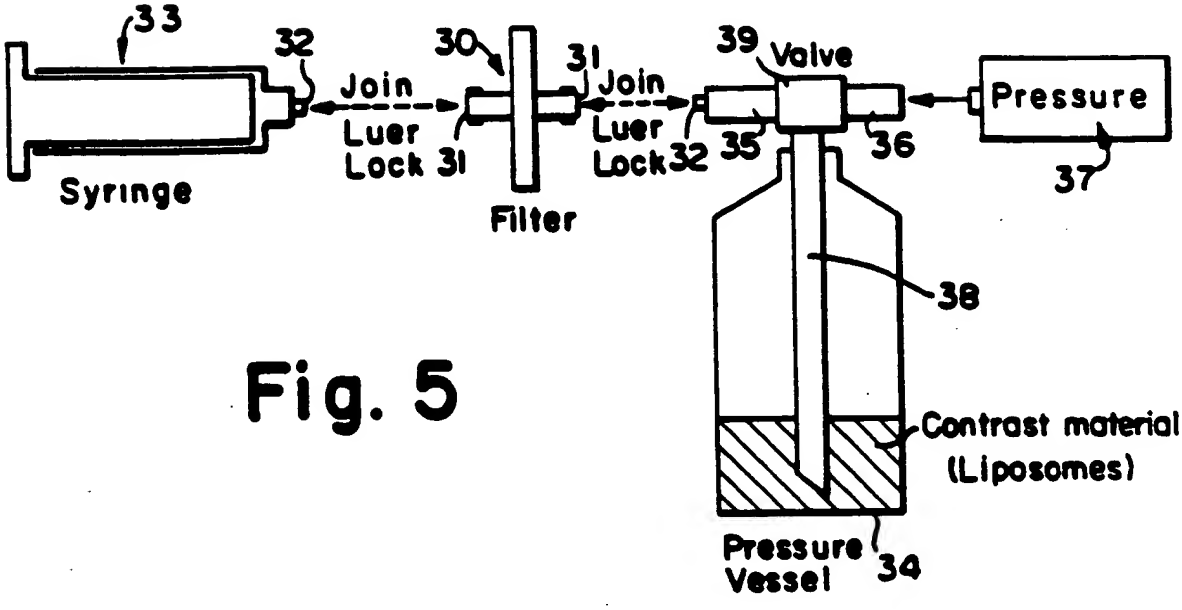


Fig. 5

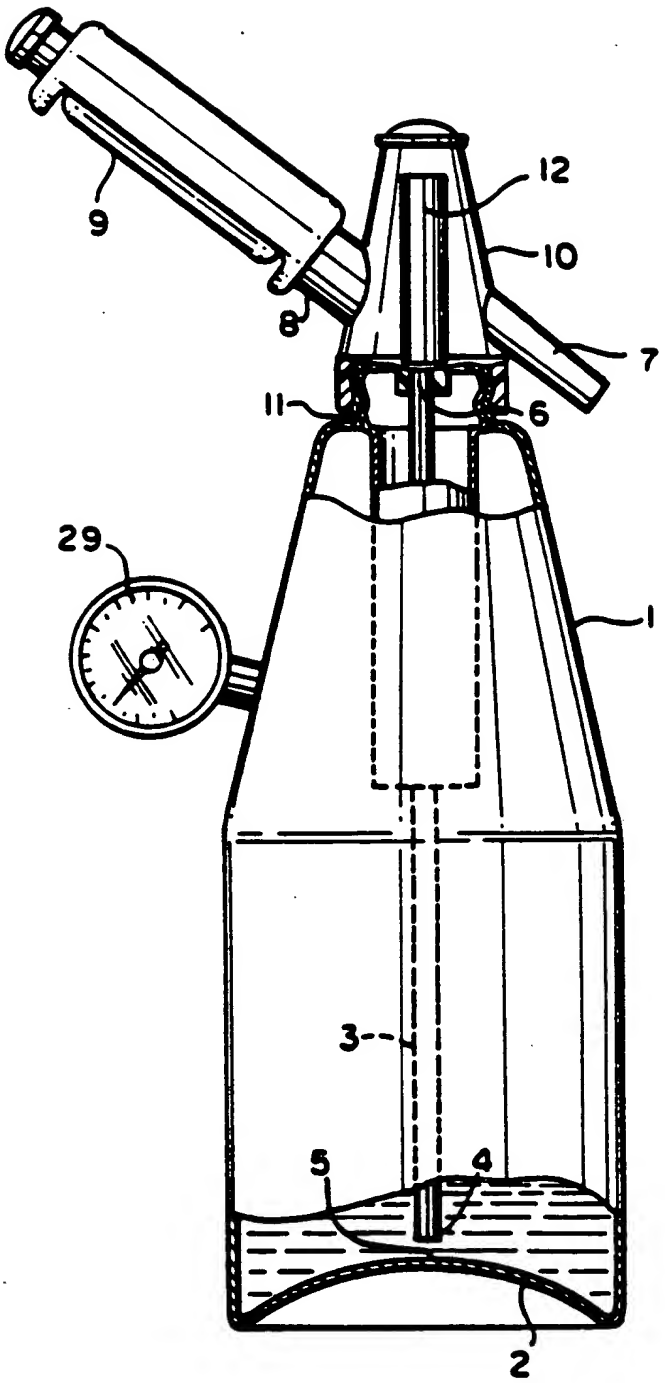


Fig. 4

Fig. 6

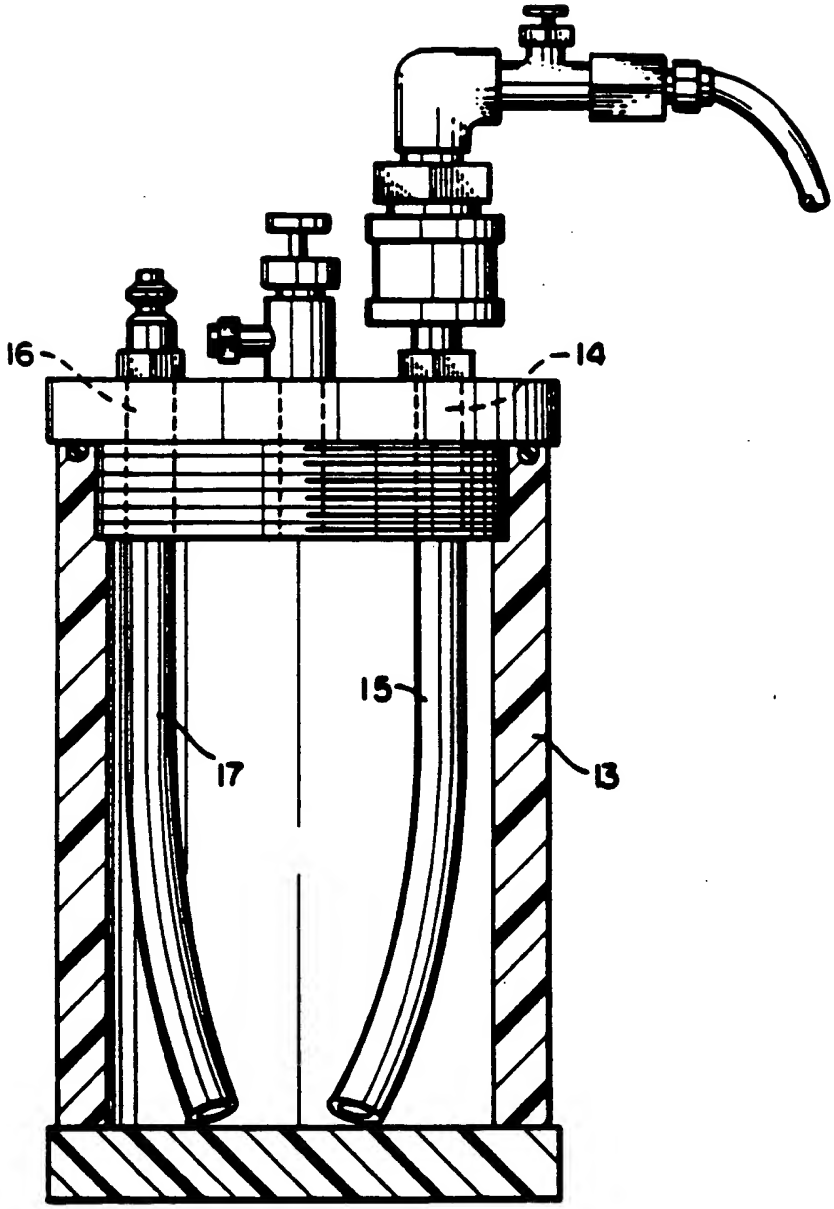


Fig. 7

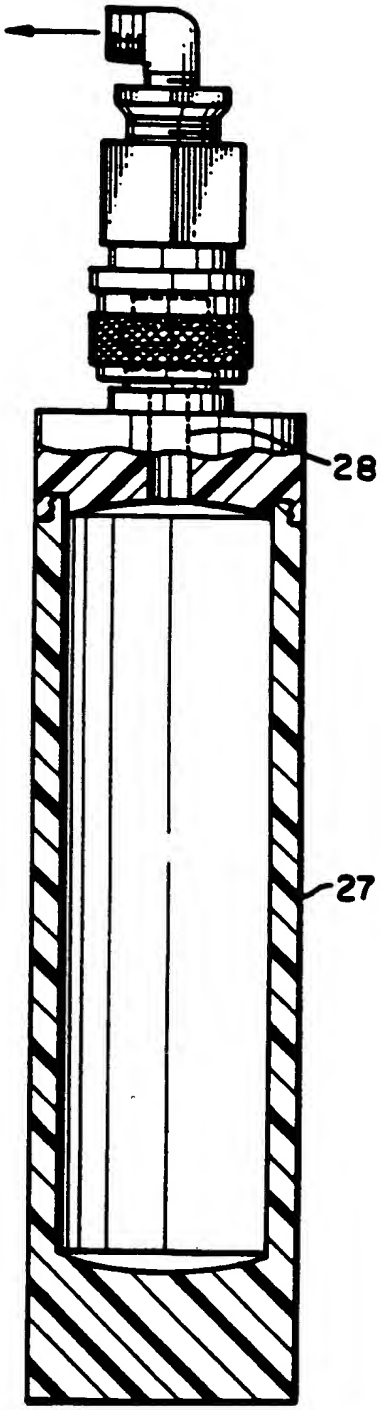


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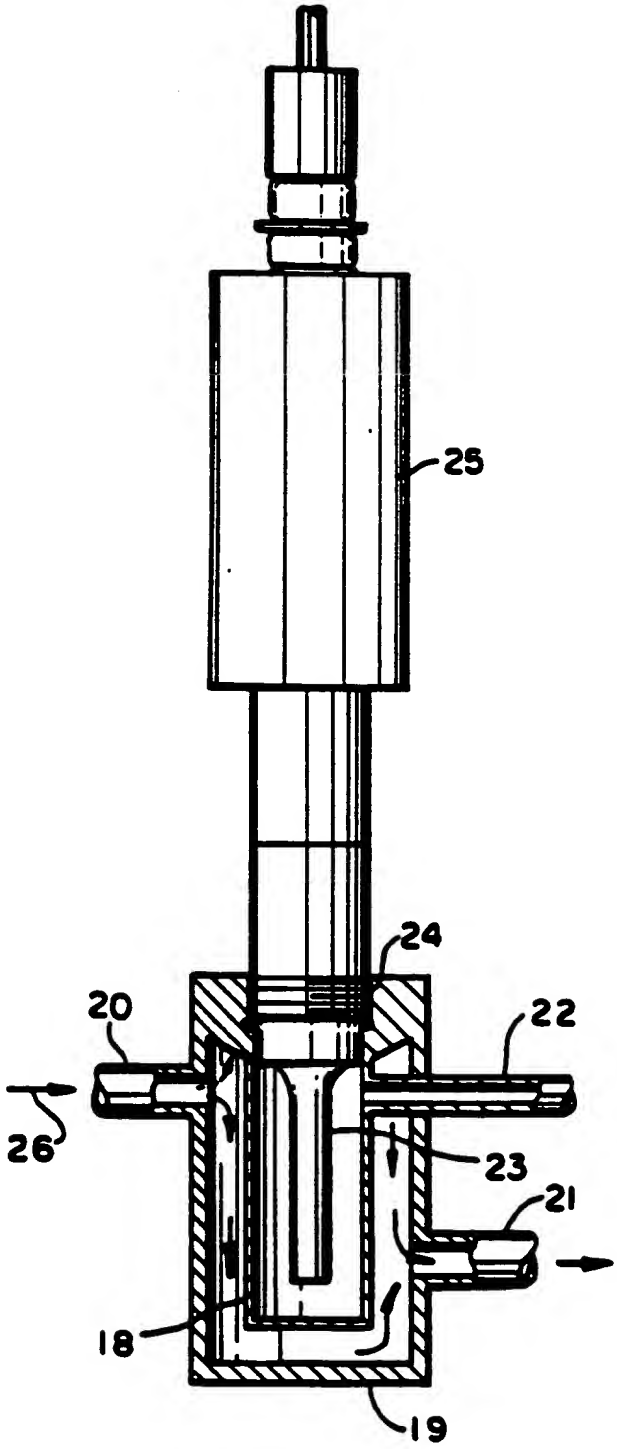
Fig. 8



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/07500

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 49/00 A61B 8/14 B01J 13/02
US Cl 424/9 128/660.01 264/4.6

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	128/662.02 654 430/152 252/350 424/9, 450, 43, 44 264/4.6,4.1 436/829

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y,P	US, A, 4,900,540 (Ryan) 13 February 1990 see entire document	1-19
y	US, A, 4,544,545 (Ryan) 1 October 1985 see entire document	1-19
y	US, A, 4,331,654 (Morris) 25 May 1982 see column 4	10,11,14
y	US, A, 3,532,500 (Priest) 6 October 1970 see column 1	5,6,11,14
y	A and E Rose, "The Condensed Chemical Dictionary," published 1966 by Reinhold Publishing, (New York) see pages 728 and 743.	7,8,11,14
y	US, A, 4,310,505 (Baldeschwieler) 12 January 1982 see column 1	1-19

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

14 February 1991

Date of Mailing of this International Search Report

18 APR 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

for Scott R. Akers Nghia Nguyen

INTERNATIONAL DIVISION

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No **
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A	US, A, 4,790,891 (Halliday) 13 December 1988 see entire document	20-50
A	US, A, 4,162,282 (Fulwyler) 24 June 1979 see entire document	20-50
A,P	US, A, 4,938,947 (Nicolau) 3 July 1990	1-50
A	M. Ostro, "Liposomes," published 1983, Marcel Dekker (New York), see pages 102-103	1-50
A	J. Am. Chem. Soc., Vol. 108, issued 1986, Hiroyuki Fukuda, "Polymer-Encased Vesicles Derived from Dioctadecyldimethylammonium Methacrylate," see pages 2321-2327	1-50
A	J. Am. Chem. Soc. Vol. 102, issued 1980, S. Regen, "Polymerized Vesicles," see pages 6638-6640	